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CD4+CD25+ LYMPHOCYTE SUBSETS IN CHRONIC GRAFT VERSUS HOST DISEASE PATIENTS UNDERGOING EXTRACORPOREAL PHOTOCHEMOTHERAPY

P. RUBEGNI, P. SBANO, G. CEVENINI[1], M.G. PERARI[2], G. MAROTTA[3], M. RISULO, M.R. CARCAGNÌ, G. D'ASCENZO, G. DE ALOE and M. FIMIANI

Department of Clinical Medicine and Immunological Sciences - Section of Dermatology; [1]Department of Surgery and Bioengineering; [2]Section of Respiratory Diseases; [3]Division of Hematology and Hematopoietic Stem Cell Transplantation; University of Siena, Italy

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The first three Authors contributed equally to the paper

Extracorporeal photochemotherapy (ECP) has been used successfully for the treatment of chronic Graft versus Host Disease (cGvHD). However, the mechanism by which ECP exerts its protective effects remains elusive. Some recent observations have suggested a possible role of certain subsets of T lymphocytes with immunosuppressive properties (T-regulatory cells) that coexpress CD4 and high levels of the interleukin-2 receptor chain: CD4+CD25+ T lymphocytes. We studied whether ECP affects the percentage of these cells in the peripheral blood of patients with cGvHD. The study population consisted of 14 patients with cGvHD refractory to systemic steroids. On enrolment in each cycle of ECP, patients underwent clinical examination, blood chemistry analysis and other instrumental procedures to document and assess involvement of the various organs and systems. For cytofluorimetric identification and phenotyping of CD4+CD25+ T lymphocytes, peripheral blood samples were collected in EDTA anticoagulant before ECP, after 48 hours, and after 6 and 12 months from the start of treatment. The 14 patients in this study received a total of more than 300 cycles of ECP, with only minor side effects. The clinical outcome was negative in 2 patients and positive in 12 patients. Within-subject analysis indicated that the percentage of CD4+CD25+ T lymphocytes before ECP and after 12 months of treatment was significantly increased. Our study confirms that changes in the percentage of CD4+CD25+ T cells induced by ECP could be a central aspect in the cascade of immune events leading to the immunological and clinical effects of this treatment in patients with cGvHD.

Chronic graft versus host disease (cGvHD) is still a severe complication of allogeneic hematopoietic stem cell transplants (HSCT) and is responsible for a high percentage of deaths in HSCT patients (1). Extracorporeal photochemotherapy or photopheresis (ECP), a procedure originally introduced by Edelson in 1987 to treat Sèzary syndrome, has recently proved to be effective in the treatment of several autoimmune/autoreactive diseases, such as allograft rejection and GvHD (2-4). Briefly, it consists of extracorporeal exposure of peripheral blood mononuclear cells to photoactivated 8-methoxypsoralen (8-MOP) and

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Mailing address: Prof. Pietro Rubegni,
Department of Clinical Medicine and Immunological Sciences,
Section of Dermatology, University of Siena,
Policlinico "Le Scotte", Viale Bracci, 53100 Siena, Italy
Tel: ++39 0577 585420 Fax: ++39 0577 44238
e-mail: rubegni@unisi.it

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subsequent re-infusion into the patients. The exact mechanism of action of ECP in GvHD is still not fully understood. Several investigations suggest that ECP modulates the immune system, inducing apoptosis of phototreated lymphocytes, maturation of monocytes into dendritic cells and restoration of Th1/Th2 balance (5). However, a clear overall picture of the immunological steps involved in the therapeutic effect of ECP has yet to emerge. Some recent observations have suggested a possible role of certain subsets of T lymphocytes that co-express CD4 and high levels of the interleukin-2 receptor chain (CD25)(6). These cells, the development and function of which is under control of the transcription factor FoxP3, are able to suppress polyclonal T (CD4 and CD8) cell activation in a contact dependent fashion, resulting in the inhibition of IL-2 gene transcription and inducing tolerance to alloantigens via co-stimulatory blockade (7).

However, the suppressive mechanisms induced by CD4+CD25+ regulatory T lymphocytes have not been completely elucidated. While the requirement for a direct cell to cell contact is well documented for regulatory T cells to trigger their immunosuppressive activity on their target cells, the role of inhibitory cytokines such as tumor growth factor b (TGF-b) and interleukine-10 (IL-10) is still being explored. FoxP3, mechanisms of suppression of T effector cells, TGF-beta and IL1In particular, it has been demonstrated in a mouse model that infusion of these CD4+CD25+ T lymphocytes (T-regulatory cells) in the inoculum of hematopoietic stem cells for bone marrow transplantation (BMT) prevented the onset of GvHD by suppressing polyclonal T cell activation (7). Moreover, Lamioni et al recently reported a significantly greater number of CD4+CD25+ T lymphocytes in the blood of transplant patients with organ rejection treated with ECP, compared to those not treated with ECP (8). On this basis, we studied a series of patients with steroid-refractory cGvHD to determine whether ECP affected the percentage of CD4+CD25+ T lymphocytes in peripheral blood, whether any change in the number of CD4+CD25+ cells in peripheral blood was correlated with clinical response to ECP and, if so, in what way.

MATERIALS AND METHODS

Patients

Table I shows the data regarding patients before ECP. Informed consent for enrolment in the study was obtained from all patients, as was the approval of the local ethics committees. The study population consisted of 14 cGvHD patients (9 male, 5 female), with a mean age of 32 years (range 19-58 years) (Table I). All were refractory to systemic steroids. Chronic GvHD was diagnosed on the basis of clinical, laboratory and histological data, according to published criteria (4).

Three identical UVAR photopheresis units (Therakos Inc., West Chester, PA, USA) were used for ECP, as previously reported (4). In each treatment cycle, 240 ml buffy coat and 300 ml plasma were obtained in special bags by standard leukapheresis and diluted in 200 ml saline. Solution containing 100,000 ng 8MOP (Gerot Pharmazeutika, Vienna, Austria) was added directly to the bags. The bag contents were run as a thin layer through a special plastic device irradiated with UVA (2 J/cm^2) for 90 min. The treated cells were then immediately re-infused. The patients underwent two treatment sessions on consecutive days every two weeks for the first 3 months and then two sessions on consecutive days every three weeks for a further 3 months. In the following 6 months, the patients in whom a response to ECP was registered underwent two treatment sessions on consecutive days every four weeks.

Cytofluorimetric identification and phenotyping of CD4+CD25+ T lymphocyte subset

For the identification and phenotyping of the CD4+CD25+ T lymphocyte subset, peripheral blood samples were collected in EDTA anticoagulant before ECP (*time 0*), after 48 hours (*time 1*), after 6 months (*time 2*) and after one year (*time 3*) from the start of treatment. Whole blood aliquots (50 mL) were labeled with 20 µL monoclonal antibodies (mAbs) for 15 min in the dark at room temperature. Erythrocyte lysis was performed using a FACS lysing solution (Becton Dickinson, San Jose, CA, USA). The following mAbs were used: IgG FITC+ IgG PE, CD3 FITC+ CD4 PE, CD3 FITC+ CD8 PE, CD25 FITC, CD25 FITC+ CD4PE and CD3 FITC CD19 PE (Becton Dickinson, San Jose, CA, USA). All samples were analysed using a dual-laser flow cytometer (FACScalibur, Becton Dickinson, San Jose, CA, USA) with the Cell-Quest software program. Lymphocytes ~~are~~ were identified and selected on the basis of two physical parameters: forward scatter (FSC) and side scatter (SSC). The percentage of mature T lymphocytes was determined (CD3+) in the gate we identified. After gating T cells for CD3 expression, the percentages of CD3+CD4+ and CD3+CD8+ and B lymphocytes were measured. At least 10,000 gated CD3+ events were sampled. The percentage of the CD4+CD25+ subset was calculated from the gate of lymphocytes given by FSC x SSC and the gate given by the FL1/FL2 dot-plot.

Table I. *Study population.*

Case no.	Age	Sex	aGvHD	cGvHD type	Clinical involvement					
					Skin	Oral mucosa	Ocular mucosa	Liver	Tcp	Lung
1	37	M	Yes	P	Yes	No	No	Yes	Yes	No
2	56	M	Yes	P	No	Yes	Yes	No	No	Yes
3	51	F	Yes	Q	Yes	Yes	Yes	Yes	No	No
4	25	M	Yes	Q	Yes	No	No	Yes	No	Yes
5	19	M	Yes	P	No	Yes	No	Yes	No	No
6	43	M	No	D	Yes	Yes	No	Yes	No	No
7	42	F	Yes	P	Yes	Yes	Yes	No	No	No
8	35	M	Yes	P	Yes	Yes	Yes	No	No	No
9	45	M	Yes	P	Yes	Yes	No	No	Yes	No
10	58	M	Yes	Q	No	Yes	No	Yes	Yes	No
11	32	M	No	D	Yes	Yes	Yes	Yes	No	No
12	37	F	No	D	Yes	Yes	Yes	Yes	No	No
13	32	F	Yes	P	Yes	Yes	Yes	Yes	Yes	No
14	58	F	Yes	P	No	No	No	Yes	Yes	No

P: progressive; Q: quiescent; D: de novo; Tcp: thrombocytopenia

Statistical analysis

Multivariate analysis of variance (MANOVA) for repeated measures was performed in order to investigate possible differences in the percentages of T lymphocytes after the first cycle of ECP (*time 1*), 6 months (*time 2*) and 12 months (*time 3*) from the start of ECP in partial and complete responders to ECP. This statistical procedure permitted evaluation of between-subject effects, i.e. multivariate differences between full and partial responders and stabilizations. Statistical analysis was performed using the SPSS statistical package.

RESULTS

The 14 patients in this study received a total of more than 350 cycles of ECP, with only minor side effects. The most frequent side effect was slight hypotension resulting from volume shifts during the leukapheresis phase of treatment (4%). Hematomas at antecubital venipuncture sites were another frequent side effect. None of these side effects required interruption of treatment, and there was no evidence of cumulative toxicity. The clinical responses of patients are shown in Table II. Overall outcome was negative (progression in affected organs) in 2 patients, in whom ECP was suspended after 6 months, and positive in 12 patients. Outcome was very good (CR in all affected organs) in 5 cases and good (PR or ST in affected organs) in the other seven. Within-subject analysis indicated significant differences in the percentage of CD4+CD25+ T lymphocytes between times T0 (before ECP) and T3 (after 12 months of ECP) ($p=0.035$), and between time T1 (after the first cycle of ECP) and T3 ($p=0.02$). No differences were found for the interaction between time and type of response ($p=0.725$): in other words, no significantly different growth of CD4+CD25+ T lymphocytes was observed between non-responders, partial responders and complete responders to ECP. Regarding the other lymphocyte subpopulation studied (CD3+CD4+, CD3+CD8 and CD19+ cells), significant differences were found only in the percentage of CD3+CD8+ lymphocytes between T0 and T3 ($p=0.079$). The latter result has been widely reported in the literature concerning the use of ECP in patients with cGvHD.

DISCUSSION

The efficacy and tolerability of ECP in patients with steroid-refractory cGvHD is confirmed by the

Table II. *Results.*

Case no.	Cutaneous cGvHD	Oral mucosal cGvHD	Ocular cGvHD	Hepatic cGvHD	Thrombocytopenia	Lung cGvHD	Overall outcome
1	CR	-	-	CR	CR	-	++
2	-	CR	CR	-	-	PR	+
3	CR	PR	CR	PR	-	-	+
4	CR	-	-	PR	-	PR	+
5	-	CR	-	CR	-	-	++
6	CR	CR	-	CR	-	-	++
7	CR	PR	PR		-	-	+
8	CR	CR	CR	-	-	-	++
9	CR	CR	-	-	CR	-	++
10	-	CR	-	PR	PR	-	+
11	PR	ST	PR	ST	-	-	+
12	PR	ST	ST	PR	-	-	+
13	PR	ST	PR	P	P	-	-
14	-	-	-	P	P	-	-

CR: complete response; PR: partial response; ST: stabilization; P: progression

Table III. *CD4+ CD25+ T cell subpopulation percentages in peripheral blood of cGvHD patients treated with ECP in comparison to the CD3+ CD4+ lymphocyte population.*

Case no.	Percentage of CD4+CD25+ lymphocytes before ECP (T0)	Percentage of CD4+CD25+ lymphocytes after first cycle of ECP (T1)	Percentage of CD4+CD25+ lymphocytes after 6 months of ECP (T2)	Percentage of CD4+CD25+ lymphocytes after 12 months of ECP (T3)
1	18	16	17.1	23
2	2.7	3.4	3.8	7.5
3	4	3.7	3.5	6.5
4	13	18.3	19.1	17.5
5	9.3	10.9	12.1	14.3
6	4.8	5.1	4.2	6.2
7	18	22	22.3	25.8
8	5.9	7	20.6	18.7
9	0.4	0.3	2	0.6
10	1.3	1.2	2.1	1.65
11	15	9.6	12.4	9.2
12	10	15	17.2	15.4
13	8.7	7.9	8.2	-
14	2.4	2.1	4.6	-

present results, as it has been by other studies (2-4). A positive response to ECP was found in 12/14 patients and a complete response in all affected organs was observed in 5 of the responders. Certainly the most interesting finding that has emerged from our study is that after 12 months of ECP we observed a

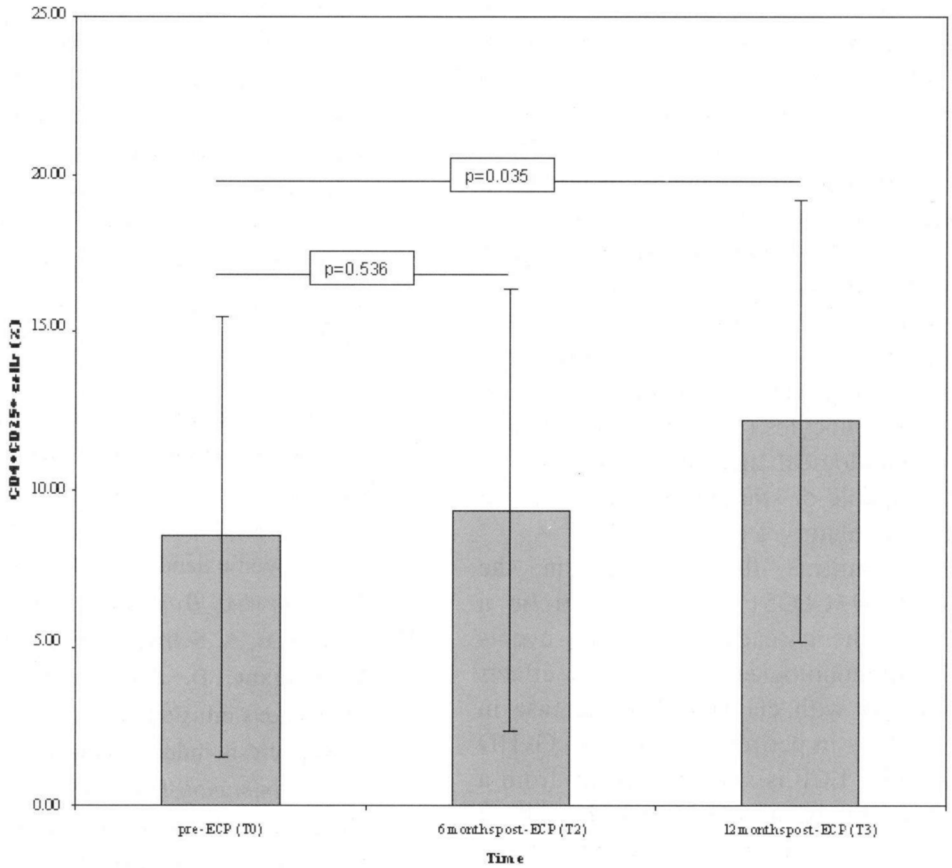

Fig. 1. *CD4+CD25+ lymphocytes are increased in the peripheral blood of cGvHD patients after ECP. The bars show the mean percentages of CD4+CD25+ T cells in 12 cGvHD patients before ECP (T0), after 6 months (T2) and after 12 months (T3) from the start of ECP.*

significant increase in the percentage of CD4+CD25+ T cells in 11 out of 12 responsive patients. Indeed in this case, within-subject analysis showed significant differences in the percentage of CD4+CD25+ T cells before and after therapy (*Time 0* versus *Time 3*).

As far as the relationship between clinical response to ECP and change in the percentage of CD4+CD25+ T lymphocytes is concerned, our small number of cases, and especially the very low number of non-responders (2/14) (who also showed a contradictory trend in CD4+CD25+ T cell percentages), exclude any statistical analysis that could demonstrate or refute a correlation between the changes in CD4+CD25+ T cell percentage and response to ECP. It was also not possible to carry out a control study in the two non-responsive patients over 12 months of treatment, as the high costs of ECP could not be justified in the treatment of non-responsive patients. However, the fact that all 12 patients who responded to ECP showed an increased percentage of CD4+CD25+ T cells after 12 months of therapy seems to indicate that ECP can immunologically influence the population of regulatory T cells, in line with recent data in the literature (8-9). Within-subject statistical analysis did not show significant changes in the percentage of CD4+CD25+ cells in circulation before (*Time 0*) and after the first cycle of ECP (*Time 1*, 48 h later). Our results therefore show that at least 48 hours must pass after ECP before any significant change in the percentage of CD4+CD25+ cells can be observed.

The immunological mechanism leading to this ECP-induced activation of CD4+CD25+ lymphocytes is certainly complex and it is difficult

to place this finding into what is already known of the complex mechanism of action of ECP in cGvHD. However, some speculation is possible. It has been shown that CD4+CD25+ T cells proliferate vigorously when stimulated with large numbers of dendritic cells (DCs) and/or apoptotic cells in an antigen- and costimulation-dependent fashion (10). Two well-known effects of ECP are: a) induction of rapid and massive apoptosis of alloreactive lymphocytes and infusion of these cells into the bloodstream (11); b) differentiation of monocytes into immature DCs that subsequently mature into potent IL-10 secreting APCs following stimulation with apoptotic lymphocytes (12). This suggests that both these immunological mechanisms exerted by ECP could be capable of stimulating production of antigen-specific regulatory T cells (12-13).

Our study confirms that changes in the percentage of CD4+CD25+ T cells could be a central aspect in the cascade of immune events leading to the immunological and clinical effects of ECP in subjects with cGvHD. The increase in CD4+CD25+ T cells in peripheral blood of cGvHD patients induced by ECP is very interesting from a therapeutic viewpoint, because these cells have been shown to be capable of improving cGvHD, without affecting the graft versus tumor effect, which is fundamental in preventing leukaemia/lymphoma relapses (6-7, 14). This particular selective effect, together with the well-known safety of ECP (absence of immunosuppression), suggest that this therapy will be increasingly used and could become a first-line treatment in patients with cGvHD.

REFERENCES

1. **Horwitz M.E. and K.M. Sullivan.** 2006. Chronic graft-versus-host disease. *Blood Rev. 20:15.*
2. **Foss F.M., G. Gorgun and K.B. Miller.** 2002. Extracorporeal photopheresis in chronic graft-versus-host disease. *Bone Marrow Transplant 29:719.*
3. **Dall'Amico R. and C. Messina.** 2002. Extracorporeal photochemotherapy for the treatment of graft-versus-host disease. *Ther. Apher. 6:296.*
4. **Rubegni P., A. Cuccia, P. Sbano et al.** 2005. Role of extracorporeal photochemotherapy in patients with refractory chronic graft-versus-host disease. *Br. J. Haematol. 130:271.*
5. **Fimiani M., M. Di Renzo and P. Rubegni.** 2004. Mechanism of action of extracorporeal photochemotherapy in chronic graft-versus-host disease. *Br. J. Dermatol. 150:1055.*
6. **Cohen J.L., A. Trenado, D. Vasey, D. Klatzmann and B.L. Salomon.** 2002. CD4(+)CD25(+) immunoregulatory T Cells: new therapeutics for graft-versus-host disease. *J. Exp. Med. 196:401.*
7. **Taylor P.A., C.J. Lees and B.R. Blazar.** 2002. The infusion of *ex vivo* activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality. *Blood 99:3493.*
8. **Lamioni A., F. Parisi, G. Isacchi, E. Giorda, S. Di Cesare, A. Landolfo, F. Cenci, G.F. Bottazzo and R. Carsetti.** 2005. The immunological effects of extracorporeal photopheresis unraveled: induction of tolerogenic dendritic cells *in vitro* and regulatory T cells *in vivo. Transplantation 79:846.*
9. **Maeda A., A. Schwarz, K. Kernebeck, N. Gross, Y. Aragane, D. Peritt and T. Schwarz.** 2005. Intravenous infusion of syngeneic apoptotic cells by photopheresis induces antigen-specific regulatory T cells. *J. Immunol. 174:5968.*
10. **Kleinclauss F., S. Perruche, E. Masson, M. de Carvalho Bittencourt, S. Biichle, J.P. Remy-Martin, C. Ferrand, M. Martin, H. Bittard, J.M. Chalopin, E. Seilles, P. Tiberghien and P. Saas.** 2006. Intravenous apoptotic spleen cell infusion induces a TGF-beta-dependent regulatory T-cell expansion. *Cell Death Differ. 13:41.*
11. **Di Renzo M., P. Rubegni, P. Sbano, A. Cuccia, C. Castagnini, G. Pompella, A.L. Pasqui, P.L. Capecchi, A. Auteri, F. Laghi Pasini and M. Fimiani.** 2003. ECP-treated lymphocytes of chronic graft-versus-host disease patients undergo apoptosis which involves both the Fas/FasL system and the Bcl-2 protein family. *Arch. Dermatol. Res. 295:175.*
12. **Spisek R., Z. Gasova and J. Bartunkova.** 2006. Maturation state of dendritic cells during the extracorporeal photopheresis and its relevance for the treatment of chronic graft-versus-host disease. *Transfusion 46:55.*
13. **Ferguson T.A., P.M. Stuart, J.M. Herndon and T.S. Griffith.** 2003. Apoptosis, tolerance, and regulatory T cells--old wine, new wineskins. *Immunol. Rev. 193:111.*

14. **Edinger M., P. Hoffmann, J. Ermann, K. Drago, C.G. Fathman, S. Strober and R.S. Negrin.** 2003. CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. *Nat. Med. 9:1144.*